

## Physiological traits and metabolic profiles of contrasting rice cultivars under mild salinity stress during the seedling stage

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### Abstract

This study aimed to investigate the effects of mild salinity stress on some physiological traits and metabolic profiles of rice during the seedling stage. Three rice cultivars differing in salinity stress response, 'KDML105' (salt-sensitive, local Thai jasmine rice), 'IR29' (salt-sensitive rice), and 'Pokkali' (salt-tolerant rice), were subjected to mild salinity stress by flood-irrigated with 40 mM NaCl. Salinity stress significantly reduces the growth and biomass of the sensitive cultivars. Electrolyte leakage in 'KDML105' and 'IR29' also increased significantly while the relative water content (RWC) decreased. However, salinity stress did not affect growth, electrolyte leakage, and RWC in the tolerant cultivar. Salinity significantly increased Na<sup>+</sup>/K<sup>+</sup> ratio in shoot and root of all cultivars. Nevertheless, the increase of Na<sup>+</sup>/K<sup>+</sup> ratio in 'KDML105' and 'IR29' was higher than that of 'Pokkali'. The metabolic profile analysis of 'KDML105' showed that uracil, L-proline, malonate, L-carnitine, L-lactate, and guanine were significantly increased, but the levels of dihydroxyacetone and glycine were significantly decreased under salinity conditions. In 'IR29', L-proline, melatonin, gamma-aminobutyrate, and acetate were significantly increased. In 'Pokkali', however, sugar alcohols and sugars were slightly increased when exposed to salinity conditions. The metabolic pathway analysis revealed one involved metabolic pathway of 'KDML105': aminoacyl-tRNA biosynthesis, and three involved those of 'IR29': arginine proline metabolism, sulfur metabolism, and butanoate metabolism. The difference in the metabolic profile of the salt-tolerance salt-sensitive cultivars provides new insights to identify additional novel biomarkers that will help better understand the mechanisms for salt tolerance in rice.

**Keywords:** 1H-NMR; metabolic profiles; physiological traits; rice; salinity stress

### Introduction

Soil salinity is one of the main factors convincing environmental problems worldwide, which limit crop productivity. Approximately 33% of the agricultural area is affected by soil salinity, and around 52 million ha in South Asia is salt-affected (Mandal *et al.*, 2018). Rice is one of the most important food crops consumed by people in almost half of the world. Rice production, however, is largely limited by salt stress conditions because

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of its susceptibility, especially at the young seedling and reproductive stages, compared to the vegetative stage. NaCl concentrations as low as 50 mM could be considered lethal at the seedling stage (Mishra *et al.*, 2020). The rice yield (Hashemi variety) was reduced up to 50% when treated with 8 dSm<sup>-1</sup> salinity (Rad *et al.*, 2011). Nevertheless, different rice cultivars show contrasting abilities in responding to salinity stress (Pattanagul and Thitisaksakul, 2008).

Plant resistance to soil salinity is a complex quantitative trait that involves many physiological and biochemical changes (Hanin *et al.*, 2016). To cope with salt stress, plants have evolved two significant types of resistance mechanisms based on either limiting the entry of salt by the roots (avoidance) or controlling its concentration and distribution (tolerance), with most plants using a combination of the two mechanisms (Hanin *et al.*, 2016). Salinity can induce water stress as it increases the osmotic pressure of the soil solution (osmotic phase), as well as excessive internal ion concentration (ionic phase), causing growth and productivity reduction (Reynolds and Tuberosa, 2008). Na<sup>+</sup>-specific damage is associated with the accumulation of Na<sup>+</sup> in leaf tissues. A high concentration of Na<sup>+</sup> in the shoot can cause a range of osmotic and metabolic problems in plants (Tester and Davenport, 2003). Homeostasis of K<sup>+</sup> and Na<sup>+</sup> ions and the K<sup>+</sup>/Na<sup>+</sup> ratio are vital for plant tolerance to many stresses, which is indicated by the fact that high concentrations of Na<sup>+</sup> in the cytoplasm are toxic for metabolically active cells, and Na<sup>+</sup> toxicity is closely related to disturbances of K<sup>+</sup> function in the cell (Rejili *et al.*, 2007). Moreover, plant growth is limited because of lower relative water content (RWC), which might be mainly due to the reduction in cytoplasmic volume and the loss of cell turgor as a result of the osmotic outflow of intracellular water (Vijayakumari and Puthur, 2016).

When plants are subjected to salt stress, they undergo excess metabolic changes that impact various cellular processes. Plant cell typically accumulates a wide range of compatible solutes under salt stress to balance the osmotic pressure of ions in vacuoles, so-called osmotic adjustment (OA). In higher plants, chlorophyll content normally decreases in salt-susceptible plants; however, it was reported that it increased in salt-tolerant plants (Heidari *et al.*, 2014). Stomatal closure is one of the responses when plants are exposed to abiotic stresses, which leads to photosynthesis reduction. Disturbance of photosynthetic mechanism increases the formation of reactive oxygen species (ROS), leading to cell membrane degradation (Apel and Hirt, 2004). Plants detoxify the ROS by increasing antioxidative enzyme activities such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (GPX), which are highlighted to be the main antioxidant enzymes involved in the tolerance of plants to stresses. A comprehensive understanding of the metabolic perturbations during salt stress is essential for gaining insight into plant salt tolerance mechanisms.

Metabolomics is a powerful approach that can be used to gain insight into metabolite compositional differences in each genotype or each treatment to understand the deep mechanism of plant stress responses (Jan and Ahmad, 2019). With the development of modern instruments, more and more techniques have been used in metabolomics studies, including chromatography, mass spectrometry, and nuclear magnetic resonance (NMR). Among these methods, 1H-NMR-based metabolomics has been proven as a potential tool for plant analysis due to its capability to detect and analyze a great number of metabolites rapidly and simultaneously (Phetcharaburanin *et al.*, 2020).

Considering the tendency to salinity areas observed during the past decade, the problem of salinity stress has become even more severe. Rice breeding for salt tolerance has long been studied, but the process requires understanding which mechanisms contribute to the desired traits and which are the impact of the stress. Therefore, a study on different responses of salt-tolerant and salt-sensitive rice cultivars is needed regarding changes in physiological characteristics and metabolic shifts. Biomarkers predicting salt-tolerant mechanisms are helpful to improve the economic rice to become more tolerant. Plants exposed to severe salinity levels respond differently from mild salinity levels, which may be the detrimental result of cellular damage. Mild salinity levels, on the other hand, can induce adaptive responses that provide more insightful details about how plants cope with stress. Therefore, the present study aimed to investigate some physiological responses and metabolic profiles of three rice cultivars differing in salt-tolerant abilities under mild salinity stress at the

seedling stage. 'KDML105' is a salt-sensitive local Thai cultivar (Pamuta *et al.*, 2020). This cultivar is popularly grown and consumed because of its aromatic characteristic. 'IR29' is a high-yielding but salt-sensitive cultivar (Razzaque *et al.*, 2019). 'Pokkali', a high salt tolerance landrace, has been well adapted to the local saline environment in coastal areas in India (Gopi and Manjula, 2018). Understanding how different rice cultivars respond to salinity stress provides significant insight for improving the salinity tolerance of rice cultivated in soil salinity regions and can be used in crop breeding to improve yield performance under salinity stress.

## Materials and Methods

### *Plant materials*

Three rice cultivars, including 'KDML105' ('KD'), 'IR29' ('IR'), and 'Pokkali' ('PK'), were germinated in distilled water for seven days. The seeds were sown in plastic pots (8 inches diameter, 7 inches height) containing 4.5 kg soil (silt loam; pH 6.34; organic carbon 19.06%; EC 0.30 dS/m; total nitrogen 1.07%; total phosphorus 42.50 mg/kg; total potassium 503.91 mg/kg; total calcium 7,262.64 mg/kg; total magnesium 802.43 mg/kg). Rice seedlings were watered with tap water and grown for seven days. On the 15th day, plants were divided into the control group (flood-irrigated with tap water) and the salt-stressed group (flood-irrigated with 40 mM NaCl). The plants were harvested on the 28<sup>th</sup> day. Shoot and root length, shoot and root fresh weight, and shoot and root dry weight were recorded.

### *Carbohydrate analysis*

The enzymes in the leaf samples were destroyed in a boiling water bath. Sugar was extracted with 80% (v/v) ethanol. The solution and fraction were kept at 4 °C until further analysis. The phenol-sulfuric method (DuBois *et al.*, 1956) was used to analyze the total sugar concentration. The total soluble sugar content was determined from a standard curve using a known concentration of glucose as a standard. Absorbance was measured at 490 nm with a spectrophotometer (Thermo Scientific™ GENESYS™ 20 Visible Spectrophotometer). Starch content was determined using hexokinase and glucose-6-phosphate dehydrogenase (G6PDH), with a known concentration of glucose as a standard (Madore, 1990).

### *Electrolyte leakage*

Electrolyte leakage was measured using an electrical conductivity meter described by Baninasab and Ghobadi (2011). A leaf piece (0.1 g) was submerged in 10 mL distilled water. The samples were incubated at room temperature for 24 hours. After incubation, the electrical conductivity of the solution (EC<sub>1</sub>) was read. The samples were then placed in a boiling water bath for 20 minutes, and the second EC (EC<sub>2</sub>) was read after cooling the solution to room temperature. The percentage of electrolyte leakage was then calculated as EC<sub>1</sub>/EC<sub>2</sub>.

### *Na<sup>+</sup>/K<sup>+</sup> ratio*

Na<sup>+</sup> and K<sup>+</sup> contents in both shoots and roots under control and stress conditions were examined using a flame photometer (S2; Thermo Finnigan, Waltham, MA, USA) as described previously (Pradheeban *et al.*, 2015).

### *Relative water content (RWC)*

The leaf was cut and immediately weighed to obtain fresh weight (FW). To obtain the turgid weight (TW), the leaf was floated in distilled water inside a closed petri dish for 5 hours, then wiped gently and weighed. Leaf samples were dried in an oven at 80 °C for 48 hours to obtain the dry weight (DW). Values of FW, TW, and DW were used to calculate RWC using the equation 1 (Yamasaki and Dillenburg, 1999):

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100 \quad (1)$$

#### *H<sub>2</sub>O<sub>2</sub> assay*

Approximately 0.1 g of rice leaf was extracted with four milliliters of 0.1% (w/v) trichloroacetic acid (TCA). Hydrogen peroxide levels were determined according to Sergiev *et al.* (1997). Half milliliters of the supernatant were added with 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M potassium iodine ('KI'). The absorbency of the supernatant was read at 390 nm, and the concentration of H<sub>2</sub>O<sub>2</sub> was calculated from the standard curve.

#### *Antioxidant enzyme activity*

Leaf tissue (0.2 g) was ground on ice using a mortar and pestle in 4 mL of grinding buffer: 50 mM potassium phosphate buffer (pH 7.8), 0.4 mM EDTA, 1 mM ascorbic acid, and stored at 4 °C. The extracts were filtered through cheesecloth, then centrifuged at 10,000 rpm for 1 minute (Lu *et al.*, 2009). A 1 mL aliquot of the supernatant was used as an extraction enzyme for superoxide dismutase (SOD; EC 1.15.1.1) (Beauchamp and Fridovich, 1971), catalase (CAT; EC 1.11.1.6) (Chandlee and Scandalios, 1984), guaiacol peroxidase (GPX, EC 1.11.1.7), and ascorbate peroxidase (APX; EC 1.11.1.11) assay (Nakano and Asada, 1981). The amount of protein in the extraction enzyme was investigated by the method of Bradford (1976).

#### *Metabolic profiling*

The rice leaf of the seedling was dried in the oven at 55 °C and powderized (400 mg). Four milliliters of buffer solution (1.5 M KH<sub>2</sub>PO<sub>4</sub>, 2mM NaN<sub>3</sub>, 1% TSP) were added into the powder and then sonicated for 15 minutes three times. Two milliliters of buffer solution were then added, vortexed, and sonicated for 15 minutes. The solution was centrifuged at 3,500 g at 4 °C for 35 minutes. Two milliliters of supernatant were left at 4 °C for the next step. Six hundred microliters of the solution were transferred to the 5 mm NMR tube. Fifty microliters of each sample were mixed for the quality control pod. All spectra were recorded using a Bruker Ascend-400 NMR spectrometer operating at a 400 MHz <sup>1</sup>H resonance frequency with the TopSpin software package 3.0 (Bruker Biospin, Rheinstetten, Germany). Quality control tests were performed on every ten samples. The scan was set to 64, and the temperature was set to 27 °C with the CPMG (Carr-Purcell-Meiboom-Gill) pulse train.

#### *Statistical analysis and data analysis*

The experiment was carried out using a randomized complete block design (RCBD) with six replications for each treatment. For physiological data, an independent sample T-test was used to compare the mean between the control and stressed condition of each cultivar. All means were separated at the *p* < 0.05 level using the SPSS. Two-way ANOVA was used to compare the effects of cultivar and treatment on physiological parameters. Pearson's correlation analysis was performed to assess the correlations between physiological parameters. For metabolome data, principal component analysis (PCA) was used to convert the variables of each sample into fewer dimensions. To identify the change in metabolites, orthogonal partial least squares (O-PLS) regression analysis was employed using continuous predictive data among plant samples to filter out undesirable variables in the data (Worley and Powers, 2016). For metabolite identification, Chenomx NMR Analysis Software and The Human Metabolome Database (HMDB) were used to match broad Spectral Reference Libraries to both identify and measure concentrations of metabolites observable in the NMR spectra.

## Results

### *Effects of cultivars, treatment, and their interaction on each parameter*

The different rice cultivars had a significant impact on various characteristics of the plants, including root and shoot weight, root and shoot length, electrolyte leakage, root and shoot Na<sup>+</sup>/K<sup>+</sup>, H<sub>2</sub>O<sub>2</sub>, GPX, and SOD. Treatments had a significant impact on root fresh weight, root dry weight, shoot length, electrolyte leakage, root and shoot Na<sup>+</sup>/K<sup>+</sup>. Additionally, a significant interaction between cultivar and treatment was observed in other characteristics like root fresh and dry weight, root length, electrolyte leakage, shoot Na<sup>+</sup>/K<sup>+</sup>, RWC, and SOD. However, total soluble sugar, starch, and some antioxidant enzymes (CAT and APX) were not affected by cultivar, treatment, and interaction between cultivar and treatment (Table 1).

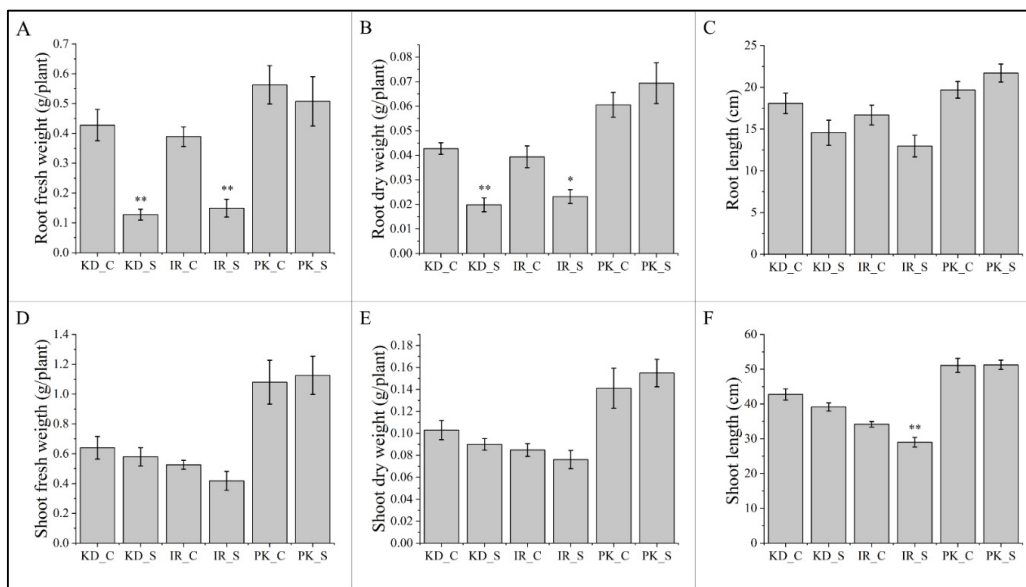
**Table 1.** Tests of between-subject effects of each physiological parameter at the seedling stage using Two-way ANOVA

Dependent variable	Cultivar (C)	Treatment (T)	C x T	R <sup>2</sup>
Root FW	13.065**	18.801**	4.925*	.673
Shoot FW	25.413**	0.294	0.359	.641
Root DW	36.033**	7.736*	6.684**	.772
Shoot DW	24.837**	0.113	1.038	.665
Root length	12.425**	3.021	3.476*	.560
Shoot length	93.665**	6.025*	1.839	.868
Total sugar	0.299	1.842	1.000	.143
Starch	0.698	1.105	1.188	.172
Electrolyte leakage	239.365**	700.37**	207.862**	.982
Root Na <sup>+</sup> /K <sup>+</sup>	5.679**	22.939**	2.647	.569
Shoot Na <sup>+</sup> /K <sup>+</sup>	15.727**	63.884**	5.908**	.819
RWC	1.057	3.355	8.301**	.451
H <sub>2</sub> O <sub>2</sub>	11.926**	0.314	1.132	.468
GPX	6.595**	0.007	0.505	.321
SOD	28.562**	2.648	11.3**	.742
CAT	1.272	0.067	0.802	.123
APX	0.407	3.703	0.506	.181

The data presented by F value with significant symbols \*\* and \* represent significance at the 1 and 5% levels, respectively.

### *Plant growth*

Salinity stress significantly decreased root fresh weight by approximately 60-70% and dry weight by approximately 45-50% in 'KDML105' and 'IR29' compared to the control group. On the contrary, root fresh and dry weight of 'Pokkali' were not affected by salinity stress. (Figure 1A, B). In the stressed condition, root length was slightly decreased in 'KDML105' and 'IR29' while slightly increased in 'Pokkali' (Figure 1C). Salinity stress slightly decreased shoot fresh and dry weight of 'IR29' and 'KDML105' but did not affect those of 'Pokkali' (Figure 1D). In addition, shoot length was significantly decreased by 17% in 'IR29', and slightly decreased in 'KDML105' when exposed to salinity stress compared to the control group. However, the shoot length of 'Pokkali' was not affected by salinity stress (Figure 1F).



**Figure 1.** Effects of salinity stress on rice seedling growth, including root fresh weight (A), shoot fresh weight (B), root dry weight (C), shoot dry weight (D), root length (E), and shoot length (F). ('KD' – 'KDML105', 'IR' – 'IR29', 'PK' – 'Pokkali', C - control, S - salinity).

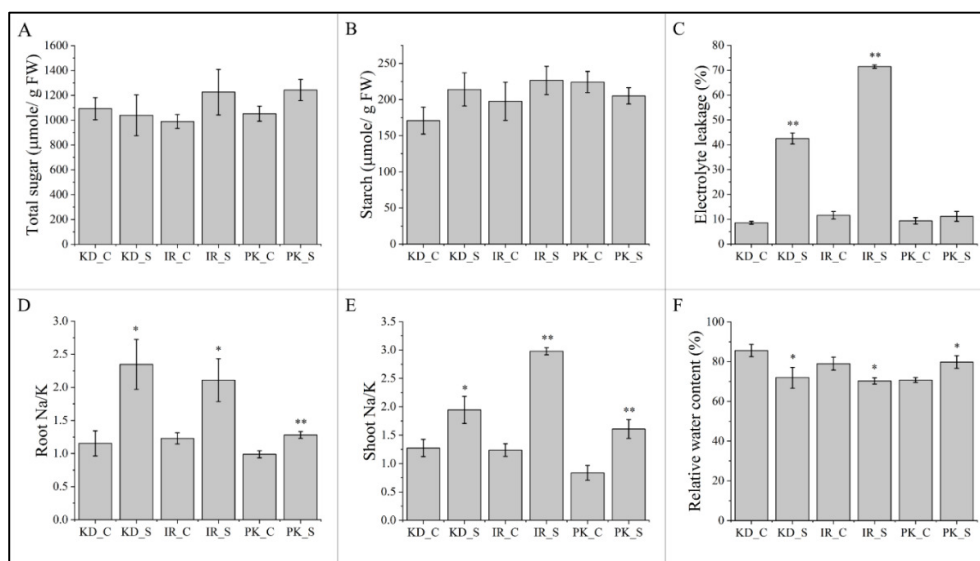
The data shows mean  $\pm$  SE. \* and \*\* represent the statistical difference between the control and stressed groups in each cultivar at  $p < 0.05$  and  $p < 0.01$ , respectively.

#### *Physiological traits*

Salinity stress had no significant effect on total soluble sugar and starch in all three cultivars (Figure 2A). Under stressed conditions, electrolyte leakage was significantly increased by approximately 4-fold in 'KDML105' and 7-fold in 'IR29', while its level remained unchanged in 'Pokkali' (Figure 2C). In addition, salinity stress significantly increased root and shoot  $\text{Na}^+/\text{K}^+$  ratio in all cultivars compared to the control group (Figure 2D). However, the  $\text{Na}^+/\text{K}^+$  ratio in the root of 'KDML105' and 'IR29' increased by 1.5- to 2- fold, while those of 'Pokkali' was slightly increased (Figure 2E). Under salinity conditions, relative water content was significantly decreased in 'KDML105' and 'IR29', but its level increased significantly in 'Pokkali' (Figure 2F).

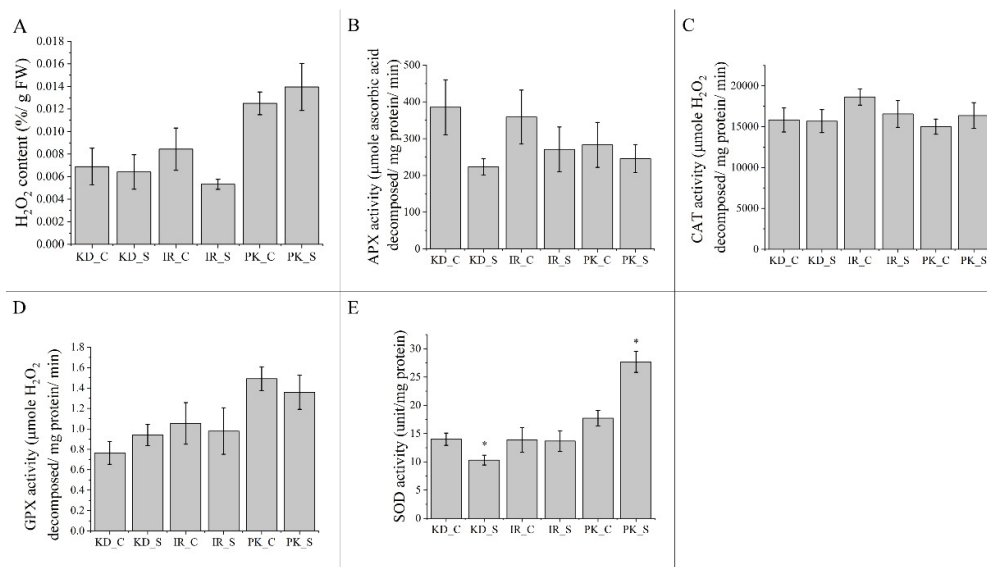
#### *Antioxidant enzyme activities*

Salinity did not affect  $\text{H}_2\text{O}_2$  content in 'KDML105' and 'Pokkali'. Interestingly,  $\text{H}_2\text{O}_2$  content in 'IR29' was slightly decreased (Figure 3A). APX enzyme activity was slightly decreased in 'KDML105' and 'IR29' when the plants were exposed to salinity stress, while its activity in Pokkali was similar to those of the control group (Figure 3B). Moreover, salinity did not affect CAT and GPX enzyme activities (Figure 3C). In addition, SOD enzyme activity in 'KDML105' was significantly decreased when subjected to salinity stress. However, SOD activity in 'IR29' was not affected by the stress, while the level was significantly increased in 'Pokkali' (Figure 3E).



**Figure 2.** Effect of salinity stress on total sugar (A), starch (B), electrolyte leakage (C), root Na<sup>+</sup>/K<sup>+</sup> (D), shoot Na<sup>+</sup>/K<sup>+</sup> (E), and relative water content (F). ('KD' - 'KDML105', 'IR' - 'IR29', 'PK' - 'Pokkali', C - control, S - salinity).

The data shows mean ± SE. \* and \*\* represent the statistical difference between the control and stressed groups in each cultivar at p < 0.05 and p < 0.01, respectively.



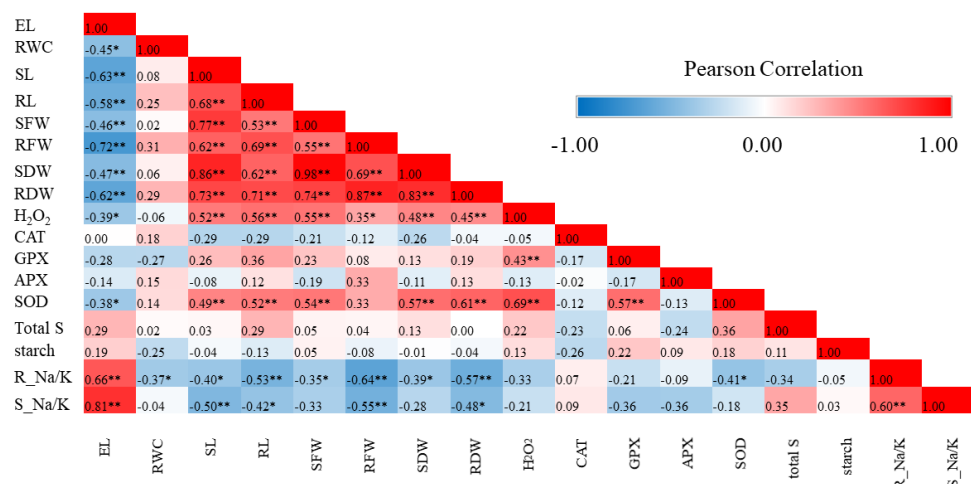
**Figure 3.** Effect of salinity stress on H<sub>2</sub>O<sub>2</sub> content (A), ascorbate peroxidase enzyme activity (APX) (B), catalase enzyme activity (CAT) (C), guaiacol peroxidase enzyme activity (GPX) (D), and superoxide dismutase enzyme activity (SOD) (E). ('KD' - 'KDML105', 'IR' - 'IR29', 'PK' - 'Pokkali', C - control, S - salinity).

The data shows mean ± SE. \* and \*\* represent the statistical difference between the control and stressed groups in each cultivar at p < 0.05 and p < 0.01, respectively.

*The correlation among physiological traits*

The correlation among 18 physiological traits of three rice cultivars under control and stressed conditions was examined using Pearson's correlation analysis (Figure 4). Soil electrical conductivity showed a positive correlation with Na<sup>+</sup>/K<sup>+</sup> and electrolyte leakage, but a negative correlation with root fresh weight. A

highly significant negative correlation was found between electrolyte leakage, plant growth parameters, relative water content, and SOD enzyme activity. Plant growth parameters showed a positive correlation with each other, SOD enzyme activity and H<sub>2</sub>O<sub>2</sub> content, however, showed a negative correlation with Na<sup>+</sup>/K<sup>+</sup>.



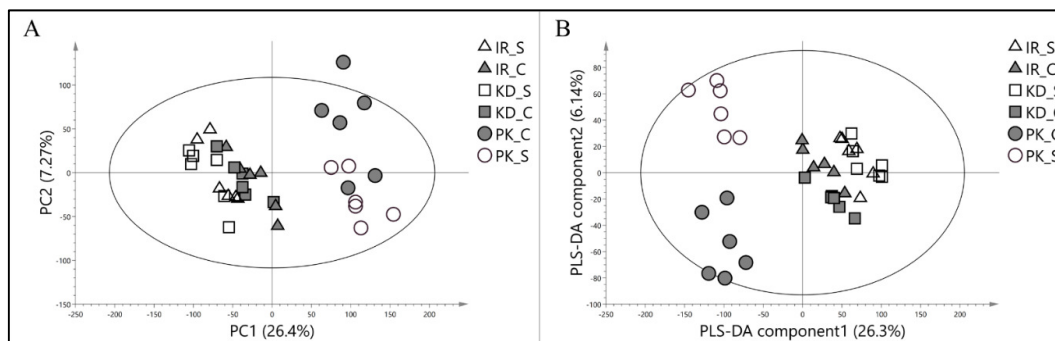
**Figure 4.** Pearson’s correlation coefficients (r values) among physiological parameters of three rice cultivars in seed seedling stage exposed to mild salinity stress. EL: electrolyte leakage, RWC: relative water content, SL: shoot length, RL: root length, SFW: shoot fresh weight, RFW: root fresh weight, SDW: shoot dry weight, RDW: root dry weight, CAT: catalase, GPX: guaiacol peroxidase, APX: ascorbate peroxidase, SOD: superoxide dismutase, total S: total soluble sugar, R<sub>Na</sub>/K: root Na<sup>+</sup>/K<sup>+</sup> ratio, S<sub>Na</sub>/K: shoot Na<sup>+</sup>/K<sup>+</sup> ratio. \*\* represents correlation that significant at the 0.01 level (2-tailed). \* represents correlation that significant at the 0.05 level (2-tailed).

*The metabolic profiles of three rice cultivars exposed to salinity stress*

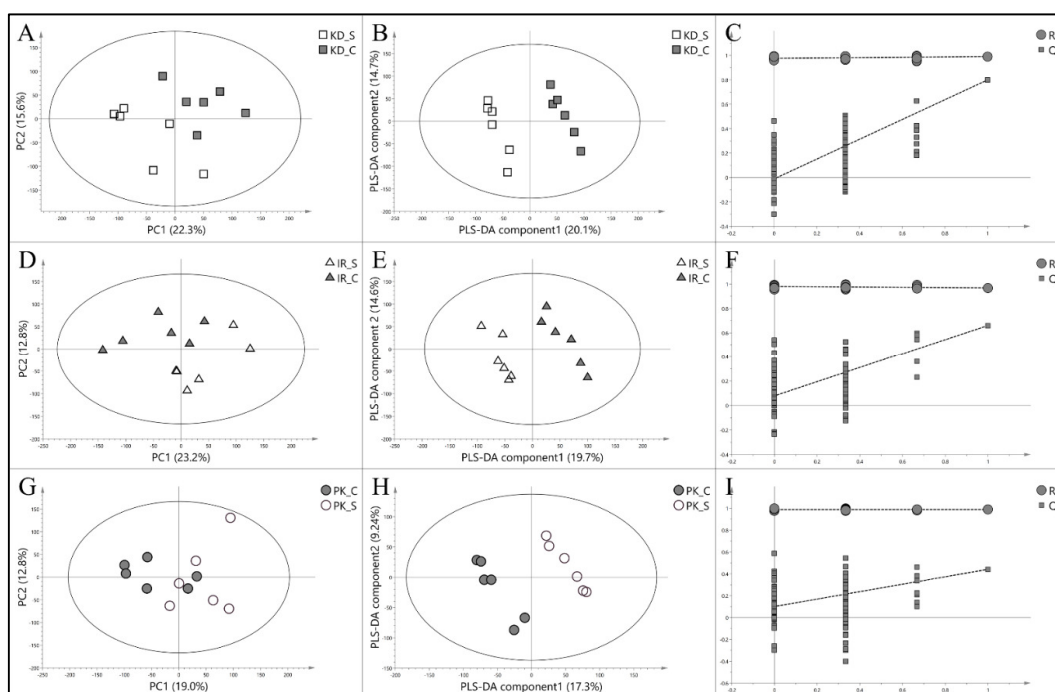
PCA was performed for an overview of the metabolomics data set, identifying outliers, and then for the exposure of any grouping. PCA is considered to focus on systematic variation through a series of NMR spectra. It displays in the calculation of a series of principal components (PCs) for each sample. The score plot was found with the first two PCs presenting 26.4% and 7.2% variance, respectively (Figure 5A). It is shown that ‘Pokkali’ was separated from the others by PC<sub>1</sub>. However, ‘Pokkali’ with control treatment cannot be detached from salinity treatment. Interestingly, ‘KDML105’ and ‘IR29’ were grouped; however, their control and salinity treatments were scattered. Then, the PCA model of each cultivar was evaluated to determine the metabolic differences between two treatments (control and stressed) in each cultivar, including ‘KDML105’ (PC<sub>1</sub>=22.3%, PC<sub>2</sub>=15.6%), ‘IR29’ (PC<sub>1</sub>=23.2%, PC<sub>2</sub>=12.8%) and ‘Pokkali’ (PC<sub>1</sub>=19.0%, PC<sub>2</sub>=12.8%) shown in Figure 6A.

PLS-DA model was established to examine the metabolic differences between the six groups. The PLS-DA score plot displayed a good separation between the ‘Pokkali’ group and the others (Figure 5B). Then, PLS-DA models with satisfactory discriminating ability were established to assess the metabolic differences between two treatment groups (control and stressed) of each cultivar (Figure 6B). According to the score plot of the PLS-DA model, control and stressed groups of all cultivars were clearly separated. The parameters for describing the PLS-DA models were significantly elevated (R<sup>2</sup>Y, Q<sup>2</sup> > 0.5), which suggested that the PLS-DA models were vigorous (Liu *et al.*, 2016). The validation plot (Figure 6C) demonstrated that the original PLS-

DA models were not random and overfitting, as both permuted  $Q^2$  and  $R^2$  values were significantly lower than the corresponding original values.



**Figure 5.** Representative PCA score plots ( $R^2X=0.336$ ,  $Q^2=0.219$ ) (A), and PLS-DA score plots ( $R^2X=0.324$ ,  $R^2Y=0.35$ ,  $Q^2=0.14$ ) (B), showing different clustering/grouping of samples, using different cultivars and treatments for each sample

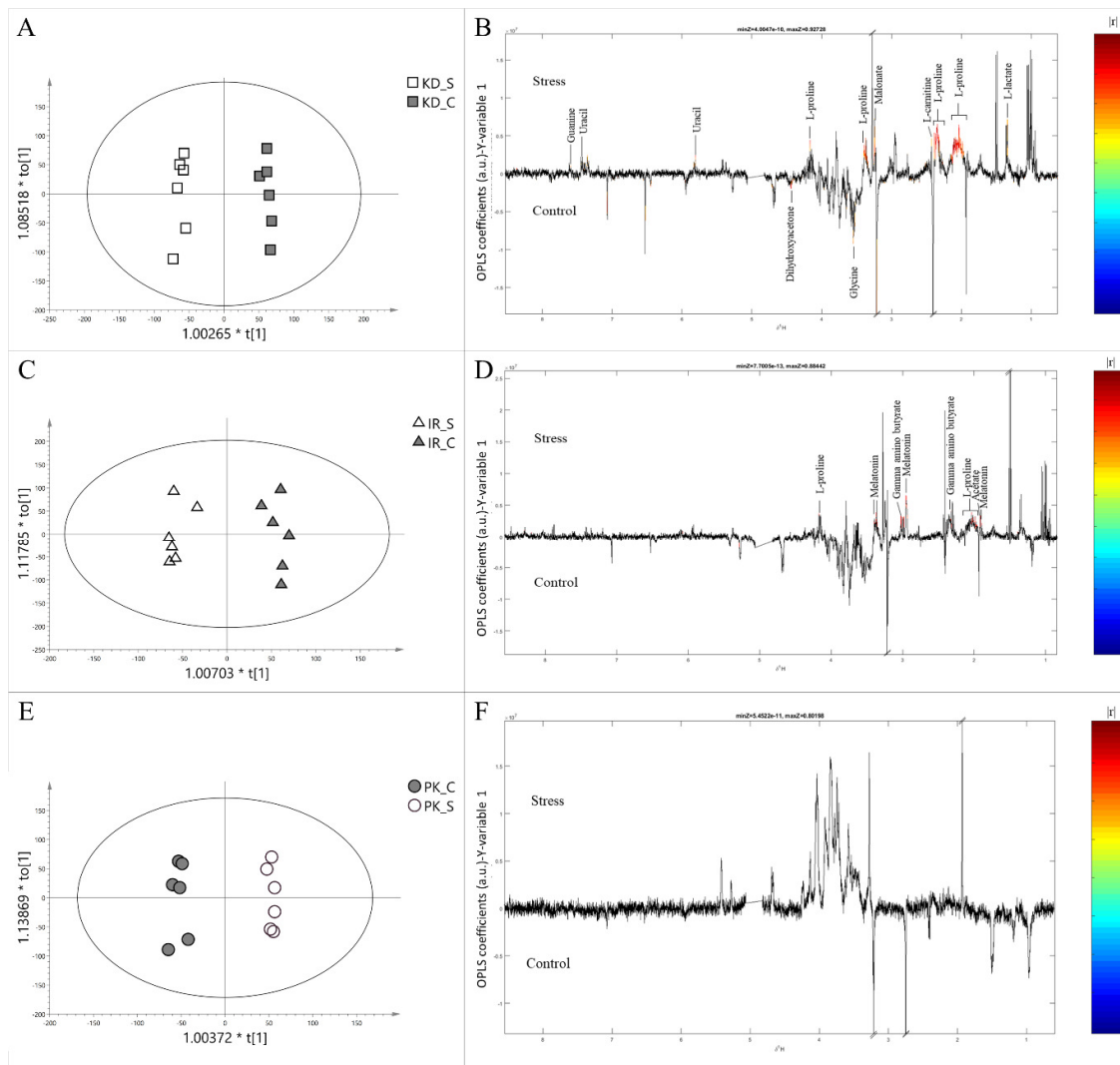


**Figure 6.** PCA score plots of ‘KDML105’ ( $R^2X=0.379$ ,  $Q^2=0.057$ ), ‘IR29’ ( $R^2X=0.359$ ,  $Q^2=0.015$ ) and ‘Pokkali’ ( $R^2X=0.318$ ,  $Q^2=-0.05$ ) (A, D, G); PLS-DA score plots of ‘KDML105’ ( $R^2X=0.347$ ,  $R^2Y=0.991$ ,  $Q^2=0.824$ ) (B), ‘IR29’ ( $R^2X=0.343$ ,  $R^2Y=0.968$ ,  $Q^2=0.716$ ) (E), and ‘Pokkali’ ( $R^2X=0.265$ ,  $R^2Y=0.989$ ,  $Q^2=0.595$ ) (H); validation plots of ‘KDML105’, ‘IR29’ and ‘Pokkali’ (C, F, I) respectively

To exclude the influence of individual variance and conduct an insight into the changed metabolites responsible for the separation between two groups, the OPLS-DA model was constructed using the first principal component and the first orthogonal component. The OPLS-DA score plots for pairwise comparison of control and stressed group samples, along with the corresponding coefficients plots depicting the major discriminators, are shown in Figure 7. In the score plot of ‘KDML105’ (Figure 7A),  $R^2X=0.347$ ,  $R^2Y=0.991$ ,  $Q^2=0.800$ , ‘IR29’ (Figure 7C),  $R^2X=0.343$ ,  $R^2Y=0.968$ ,  $Q^2=0.660$ , and ‘Pokkali’ (Figure 7E),  $R^2X=0.265$ ,

$R^2Y=0.989$ ,  $Q^2=0.441$ ), and 'Pokkali', a significant biochemical distinction between the control and stressed group was identified.

The metabolic changes in 'KDML105', 'IR29', and 'Pokkali' were reflected in the color-coded coefficient of OPLS-DA loading plots (Figure 7B). Metabolites exhibiting significant changes ( $p < 0.05$ ) were identified based on the absolute cutoff value of correlation coefficients ( $|r|$ ). In 'KDML105', the resonances assigned to uracil, L-proline, malonate, L-carnitine, L-lactate, and guanine were significantly increased, but the levels of dihydroxyacetone and glycine were significantly decreased when exposed to salinity condition. In 'IR29', the resonances assigned to L-proline, melatonin, gamma-aminobutyrate, and acetate were significantly increased under salinity stress conditions. In 'Pokkali', however, sugar alcohols and sugars were slightly increased when exposed to salinity conditions explained by the peaks in the area ( $^3H$  3.5-4.0) (Figure 7F).

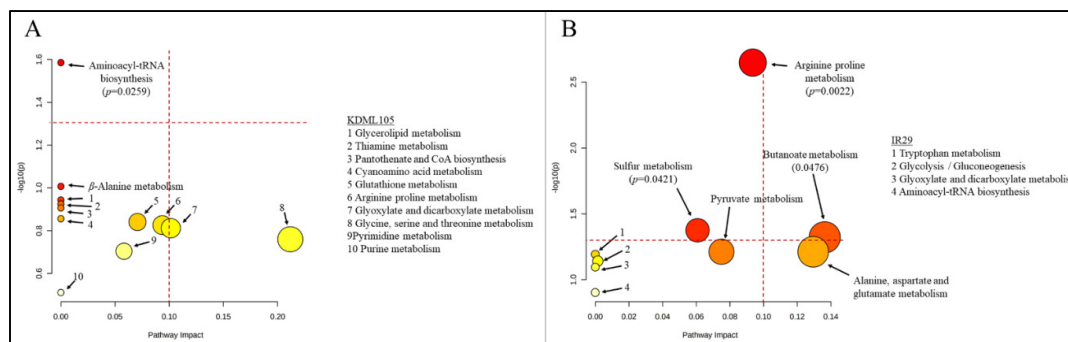


**Figure 7.** OPLS-DA score plots between control and stressed treatment of 'KDML105' ( $R^2X=0.347$ ,  $R^2Y=0.991$ ,  $Q^2=0.800$ ) (A), 'IR29' ( $R^2X=0.343$ ,  $R^2Y=0.968$ ,  $Q^2=0.660$ ) (C) and 'Pokkali' ( $R^2X=0.265$ ,  $R^2Y=0.989$ ,  $Q^2=0.441$ ) (E); OPLS-DA loading plots Bonferroni and Hogberg test ( $p < 0.05$ ).

A total of eight metabolites of 'KDML105' were considered responsible for the control and stressed treatments. All eight attributed metabolites were analysed with MetPA, and the most influenced pathways are

shown in Figure 8A and Supplementary Table 1. The most influenced metabolic pathway was ascribed a pathway impact of  $p < 0.05$ . One metabolic pathway was found to be reprogrammed: Aminoacyl-tRNA biosynthesis ( $p = 0.0259$ ). The detailed metabolites are listed in Supplementary Table 2.

In 'IR29', four metabolites were responsible for the control and stressed treatments. All four attributed metabolites were analysed with MetPA, and the most influenced pathways are shown in Figure 8B and Supplementary Table 3. The most influenced metabolic pathway was ascribed a pathway impact of  $p < 0.05$ . Three metabolic pathways were found to be changed: arginine proline metabolism ( $p = 0.0022$ ), sulfur metabolism ( $p = 0.0421$ ), and butanoate metabolism ( $p = 0.0476$ ). The detailed metabolites are listed in Supplementary Table 4.



**Figure 8.** Altered pathway analysis of 'KDML105' (A) and 'IR29' (B). The x-axis represents the pathway impact, and the y-axis represents the  $-\log_{10}(p)$ . Plots depicting computed metabolic pathways as a function of  $-\log_{10}(p)$  and pathway impact for the key differential metabolites from control and stressed treatments.

## Discussion

The salinity tolerant mechanism in plants is a complex process that generally involves two major actions. Ion exclusion is the first mechanism to reduce the accumulation of toxic  $\text{Na}^+$  within roots and leaves by controlling the  $\text{Na}^+$  loading into the xylem and  $\text{Na}^+$  retrieval from the xylem before reaching the photosynthetic tissues in the shoot (Almeida *et al.*, 2017). Tissue tolerant is the second mechanism, in which high salt concentration is found in leaves, but  $\text{Na}^+$  is compartmentalized at the cellular and intracellular level (especially in the vacuole), reducing the lethal effects of  $\text{Na}^+$  in the cytosol and driving water uptake to cells (Roy *et al.*, 2014). In the present study,  $\text{Na}^+/\text{K}^+$  in the root and shoot tissues are influenced by both rice cultivars and salt treatments. Under salinity conditions, the root  $\text{Na}^+/\text{K}^+$  of the sensitive cultivar increased by 1.5- to 2-fold compared to the control group, while those of the tolerant cultivar were slightly increased. High  $\text{Na}^+$  uptake interrupts the transport of other nutrient ions, especially  $\text{K}^+$ , leading to  $\text{K}^+$  deficiency. Many glycophytes exhibiting improved tolerance to salinity stress have a higher ability for sodium elimination, conserving low levels of  $\text{Na}^+/\text{K}^+$  ratio (Assaha *et al.*, 2017). 'Pokkali' can preserve a low  $\text{Na}^+/\text{K}^+$  ratio and shows fast growth rate under salinity conditions, making it more tolerant to stress (Walia *et al.*, 2005).

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is one of the reactive oxygen species (ROS) induced by salinity stress. Nevertheless,  $\text{H}_2\text{O}_2$  at a low level can improve plant growth parameters and rice yield production by inducing protection against subsequent severe oxidative or abiotic stress (Jira-anunkul and Pattanagul, 2021). On the other hand, a high level of ROS triggers cellular disorders such as lipid peroxidation causing cell defects such as ion leakage and cellular dysfunction (Sharma *et al.*, 2012). The cell membrane is the first component to be affected by salt stress due to the peroxidation of lipids in the membrane system resulting in increased membrane permeability (Kaya *et al.*, 2009). In rice, salinity distorts the subcellular architecture leading to plasmolysis,

increased cytoplasmic vesiculation, and damage to all types of membranes in the cell (Zheng *et al.*, 2012). In this investigation, electrolyte leakage was used as an index of lipid peroxidation. Electrolyte leakage was significantly increased in the salt-sensitive rice cultivars with NaCl stress, while its level remained unchanged in the tolerant cultivar. Similar results were also reported in the sensitive and tolerant wheat cultivars subject to NaCl stress (Radi *et al.*, 2013). Polyunsaturated fatty acids are the main membrane lipid components susceptible to peroxidation and degradation by free radicals. Plants have evolved enzymatic and non-enzymatic antioxidant defense mechanisms to control ROS levels. Our study found that 'Pokkali' generally contained the highest H<sub>2</sub>O<sub>2</sub>, GPX, and SOD compared to the others. We found that the SOD activity of the tolerant cultivar was significantly increased when exposed to salinity stress. SOD is an enzyme that catalyzes the dismutation of O<sub>2</sub><sup>-</sup> to H<sub>2</sub>O<sub>2</sub>; therefore, they constitute a frontline in the defense against ROS. A similar result has been previously described in rice grown in saline conditions (Scandalios, 2005). High salt stress increases FeSOD and CuZnSOD activities in pea plants. These activities might characterize a protective role against the assembly of O<sub>2</sub><sup>-</sup> to respond positively to salt stress (Hernández *et al.*, 2001). Maintaining high antioxidant capacity has been related to increased stress tolerance of crop plants by helping a plant protect itself against oxidative damage by rapidly scavenging the toxic levels of ROS in its cells and restoring redox homeostasis (Caverzan *et al.*, 2016). In 'KDML105', SOD activity was decreased when exposed to salinity stress; however, its metabolic profile showed that L-carnitine was increased. L-carnitine has detoxification and antioxidant roles in plant cells, which may contribute to alleviating the damaging effects of salt stress by increasing mitosis and decreasing DNA injury caused by oxidative stress reported in barley seedlings (Oney-Birol, 2019).

Generally, plants increase nutrient uptake by increasing root surface area as an adaptive mechanism under salinity stress (Arif *et al.*, 2019). Our results showed that the salt-tolerant rice cultivar could be grown without detrimental effects in the mild salinity condition, while the growth of the sensitive cultivars was heavily reduced. Root growth can be reduced by salinity up to 50% in root length density because salinity induces regulated biophysical restraints to cell wall expansion which, in turn, inhibits root expansion (Nam *et al.*, 2015). In addition, salinity induces stomatal closure, causing an increase in leaf temperature and inhibiting shoot elongation. These effects are independent of the accumulation of salts in the shoot (Rajendran *et al.*, 2009). Salinity reduces plant growth by causing physiological water stress, inhibiting cell division and expansion (Zheng *et al.*, 2012). RWC was used to measure leaf hydration status representing the osmotic tolerant ability caused by salt and drought stresses (Vijayakumari and Puthur, 2016). Under salinity conditions, the tolerance cultivar was capable of maintaining RWC, while the level in the sensitive cultivars was reduced. Sairam *et al.* (2005) reported that salinity reduced RWC in the salt-sensitive wheat genotype compared to the tolerant genotype. The salt-tolerance cultivars, Pokkali and CSR-13, maintained higher RWC under salt stress than the salt-sensitive cultivar (Pal *et al.*, 2009). One of the mechanisms to maintain RWC is compatible solute accumulation. The production and accumulation of compatible solutes, such as proline, glycine betaine, trehalose, and polyols, is crucial for maintaining osmotic pressure equilibrium in conditions of high salinity. While the process of osmotic adjustment, characterized by an increase in proline accumulation in 'IR29', was noted in plants subjected to this condition, this mechanism may not be adequate to maintain the requisite equilibrium of water in the plant system. On the other hand, in the context of the polyol pathway, the study identified an increase in the concentration of sugar and sugar alcohol upon exposure to osmotic pressure in salt-tolerant cultivar. This phenomenon is noteworthy as it serves a dual purpose of contributing to osmotic adjustment as well as aiding in the stabilization of cellular membranes.

In terms of compatible solutes, tolerant cultivar accumulates sugar which plays an important role in maintaining the overall structure and growth of plants (Rosa *et al.*, 2009). Sugar regulates plants in a very complex method because of their site-specificity. Therefore, it requires long distant signals to coordinate with physiological, developmental, and environmental alterations (Lemoine *et al.*, 2013). The accumulation of soluble carbohydrates in plants has been widely reported as a response to salinity, despite a significant decrease in net CO<sub>2</sub> assimilation (Pattanagul and Thitisaksakul, 2008). In the present study, salinity stress has no effect

on total soluble sugar and starch contents in all cultivars, whereas the metabolic profile shows an increase in glucose in 'Pokkali'. Radi *et al.* (2013) revealed that a high level of salinity stress induces the accumulation of soluble sugars in shoots and roots of tolerant and sensitive wheat cultivars. In contrast, mild stress stimulates the accumulation of soluble sugars only in the sensitive wheat cultivar. Accumulation of sugars in the plant was considered one of the most significant consequences of osmotic adjustment under salt stress. Carbohydrates may also contribute to increased membrane stabilization (Matros *et al.*, 2015). The accumulation of sugar under salt stress supported the well-established role as an osmoprotectant that stabilizes cellular membranes and maintains turgor (Singh *et al.*, 2015). Jouve *et al.* (2004) concluded that the observed variations in the endogenous content of monosaccharides might be due to a slowdown in sugar transfer from the mesophyll cells to the phloem.

Metabolomics has been applied to identify biomarkers for plant responses to abiotic stress and yield prediction. In the present study, <sup>1</sup>H NMR-based metabolomics approach was conducted to reveal metabolic differences between control and stressed treatments of three rice cultivars differing in salinity tolerant ability. Subsequent analysis of metabolite profiles of rice leaf samples under stressed conditions could discriminate the stressed group from the control group, provide a fingerprint of metabolic changes that characterized the stress responses, and highlight the potential of metabolomics analysis under stress conditions. 'Pokkali' contains different metabolic profiles from the others because of a unique set of gene transcription and translation in salt-tolerant and salt-sensitive rice (Li *et al.*, 2018). 'Pokkali' with control treatment can be detached from salinity treatment by the slight change of sugar alcohols and sugars at <sup>3</sup>H 3.5-4.0, which subsidized the density by the presence of oxygenated methylene and methine groups in their structures (Kil *et al.*, 2022). These compounds are accumulated in the tolerant cultivar in order to minimize water loss and maintain cellular integrity, so-called osmoprotectants and are characterized by their low molecular weight, high polarity, solubility, and hydrophilicity (Krasensky and Jonak, 2012). 'KDML105' and 'IR29' were grouped, however, their control and salinity treatments were scattered. Jankangram *et al.* (2013) compared leaf lamina proteomes of 'KDML105' and 'Pokkali' under high salt. The study found that although 'KDML105' contains the elevated level of salinity-related proteins that are also found in 'Pokkali', its posttranscriptional mechanisms for protein expression are not as efficient as that of 'Pokkali', revealing the basis of salt sensitivity in 'KDML105' rice cultivar. This reason may cause the different metabolic profiles between each cultivar. Li *et al.* (2018) also revealed that the translational regulation under salinity plays an important role in the salt-tolerant 'Pokkali', however, such regulation was less evident in the salt-sensitive 'IR29'. Pathway impact is a combination of the centrality and pathway enrichment results. It is calculated by adding up the importance measures of each of the matched metabolites and then dividing them by the sum of the important measures of all metabolites in each pathway. In our study, 1 unique metabolic pathway of 'KDML105' and 1 that of 'IR29' are identified from the differences of control and stressed treatments which are aminoacyl-tRNA biosynthesis and arginine proline metabolism respectively indicating their different regulation of metabolic pathway in response to salt stress.

## Conclusions

From our study, the rice cultivars responded broadly in terms of their physiological and metabolic traits and differential tolerance to salinity stress. Under mild salinity conditions, the salinity-tolerant cultivar maintained all growth parameters, while the root growth of the sensitive cultivars was decreased. Moreover, salinity stress significantly alters two traits, RWC and SOD enzyme activity, which may represent a strategy to challenge salt stress. Different rice cultivars comprise various metabolites closely related to their nutritional and functional effects. All the physiological and metabolic profiles, such as growth parameters, RWC, SOD, L-proline, melatonin, and gamma-aminobutyrate revealed significant differences between the tolerance and

sensitive cultivars, which provides insights to identify additional novel biomarkers that will help better to understand the mechanisms for salt tolerance in rice.

### Authors' Contributions

Conceptualization: W.P. and J.P.; Data curation: T.J. and M.S.; Formal analysis: T.J.; Funding acquisition: W.P.; Investigation: T.J.; Methodology: J.P., W.P. and M.S.; Project administration: W.P.; Resources: J.P. and W.P.; Software J.P.; Supervision: W.P.; Validation: J.P. and M.S.; Visualization: T.J. and M.S.; Writing - original draft: T.J.; Writing - review and editing: J.P. and W.P. All authors read and approved the final manuscript.

### Ethical approval (for researches involving animals or humans)

Not applicable.

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### Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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